Influence of Sympathetic Nervous System Activation on Secretory Immunoglobulin-A During Cold Pressor Test

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Thesis Abstract

Influence of Sympathetic Nervous System Activation on Secretory Immunoglobulin-A During Cold Pressor Test

Ghazal Mohammadi, MSc.

I investigated the response of secretory immunoglobin-A (SIg-A) to a cold pressor test (CPT). Acute physical stressors trigger the activation of the sympathetic nervous system, which may influence an immune response. Inter-individual differences in autonomic responses to acute stressors make it difficult to draw conclusions on immune system responses. My primary hypothesis was that the CPT causes an increase in SIg-A in saliva. My secondary hypothesis was that the increase in SIg-A has direct correlation to the increase in salivary α-amylase (SαA) concentrations, cardiovascular and hemodynamic parameters indicative of sympathetic activity.

Twenty participants completed a 5-min CPT, with cardiovascular parameters measured and saliva samples collected. The effect of CPT on SIg-A had a decreasing trend that was variable in individuals. The CPT caused the SαA, HR, BP to increase ($p< 0.05$). There were no correlations between SIg-A and SαA concentrations, cardiovascular and hemodynamic changes during and post CPT. Our analysis showed an inverse correlation between the SIg-A magnitude of change and its baseline value ($r = -0.60$, $p= 0.01$). Therefore, the CPT increased SIg-A in participants with a low baseline, and decreased SIg-A in participants with a high baseline. Our results suggest that the sympathetic nervous system does not affect the SIg-A concentrations and SIg-A changes according to its baseline in accordance with the law of initial values. These results will help interpret SIg-A studies, and to understand the interindividual variations in SIg-A responses.

Keywords: immune system, cold pressor test, sympathetic nervous system, secretory immunoglobin-A, salivary α-amylase.

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Special Symbols

α: Alpha

Δ1: CPT value – baseline value

Δ2: recovery value - CPT value

Acronyms

CPT: Cold pressor test

SIg-A: Secretory immunoglobulin-A

SαA: Salivary α-amylase

BP: Blood Pressure

DBP: Diastolic blood pressure

SBP: Systolic blood pressure

BPM: Beats per minute

CO: Cardiac output

HR: Heart rate

PEP: Pre-ejection period

LVET: Left ventricular ejection time

MAP: Mean arterial pressure

SV: Stroke volume

TPR: Total peripheral resistance

ICG: Impedance cardiography

1.0 Introduction

1.1 Secretory Immunoglobulin-A

The immune system's activity inside the oral cavity is indispensable. The oral cavity is one of the most likely sites for pathogenic invasions since it is continuously subjected to the external environment and foreign material through breathing or eating and drinking. The epithelium is the interface between the mucosal surface and the external environment which is rich in foreign antigens and microbial pathogens (Kraehenbuhl & Neutra, 1992). Although the epithelium works as a barrier for some pathogens, it also prevents the plasma lymphocytes such as T cells and B cells from reaching the areas that are infected or exposed to infection (Marcotte & Lavoie, 1998a). For this reason, the oral cavity has a type of first line immune system that can attenuate pathogens from entering the body.

The immune system can defend the body by two strategies: cell-mediated immunity or humoral immunity (Kubitz, Peavey, & Moore, 1986). Cell- mediated immunity is the protective function which activates cells such as T cells (Andersson, Bird, Britton, & Palacios, 1981). The humoral immunity is when secreted antibodies or other substances that have antimicrobial traits are secreted from immune cells are involved in an immune response in the cell-free body fluid or serum, which was historically called humor (Kubitz et al., 1986). The humoral defense system ensures the immunity of the mucosal membranes of the body including the oral cavity (Stone, Cox, Valdimarsdottir, Jandorf, & Neale, 1987).

An antibody or an immunoglobulin is a "Y-shaped" protein that uses a variable region to fight infections (Stone et al., 1987). Fab's variable region is a key and lock mechanism of action as the tips of Y is the paratope (lock) that is definite for one precise epitope (key) on an antigen (A. J. Macpherson, Geuking, & McCoy, 2011). By binding to the antigen, the antibody can directly neutralize the invader or indirectly by flagging out the target for the killer cells to eliminate the pathogen (Boyd, 1947).

Immunoglobulin isotopes consist of five heavy chain classes; Ig-A, Ig-D, Ig-G, Ig-E, Ig-M (Stadlmann et al., 2009). They are sub-grouped based on their location in the body, the stage of an adaptive immune response, and specialized response to specific types of antigens (Boyd, 1947). Various Igs such as Ig-A, Ig-M, and Ig-G exist in saliva that come from the salivary

glands or the peripheral blood circulation via crevicular fluid (Brandtzaeg, 2013). Ig-A is a dimer form, found in the mucus and is also a monomeric form, found in the serum of blood (Kaetzel, Robinson, Chintalacharuvu, Vaerman, & Lamm, 1991).

The hinge region is a flexible amino acid stretch functioning as the interdomain link of two "Y shaped" heavy chains of the dimer Ig-A. Ig-A antibodies are divided into two subclasses based on the size of the hinge region; Ig-A1 and Ig-A2 (A. Macpherson, McCoy, Johansen, & Brandtzaeg, 2008). IgA1 dominates the serum IgA and possess a longer hinge region, while IgA2 is predominantly in mucosal secretions with comparably shorter hinge region (Stadlmann et al., 2009).

Secretory immunoglobulin-A (SIg-A) is the first line of defense that encounters the pathogens inside the oral cavity (A. J. Macpherson et al., 2011). SIg-A is perhaps the main form of antibodies as its production rate is above all the other antibodies (Marcotte & Lavoie, 1998a). Beside saliva, SIg-A is also charged with the defense of all mucosal areas such as tears, sweat, colostrum, intestinal, genito-urinary, breast milk, gastrointestinal tract, and respiratory epithelium (Woof & Kerr, 2006). Slg-A concentration changes can be observed immediately and with direct correlation with the causative agent or the stressor because it has a half-life of 3-6 days and a high synthesis rate 66 mg/kg/day (Tobina et al., 2003).

Saliva is the key component for the SIg-A antibody transportation inside the mouth to pass the epithelium. It contains a combination of mucus and a watery substance named serous, playing a role in digestion and immunity (Mandel, 1987). Saliva consists of 99.5% water, mucus, electrolytes, antibodies, epithelial cells, glycoproteins, and enzymes (e.g. amylase and lipase) (Schneyer, Young, & Schneyer, 1972). Saliva is produced by acinar cells and secreted by clusters of acini cells in the glands salivary glands. In humans, there are three major glands responsible for saliva production the parotid, submandibular (submaxillary) and sublingual salivary glands (Edgar, 1992). These three salivary glands work together and each gland possesses a particular duct connected to the mouth and oral cavity.

Besides the major glands, there are up to a million minor salivary glands within the tongue, lips, cheeks, and palate (Catalán, Ambatipudi, & Melvin, 2012). Parotid glands are the largest salivary gland, located posterior to the mandibular ramus and anterior to the mastoid process of the temporal bone (Kraehenbuhl & Neutra, 1992). The parotid glands make about 20% of the total saliva and produce the serous type of saliva or the watery part, mostly in charge of

amylase production and aiding mastication and digestion (Lashley, 1916). The saliva from the parotid glands enters the oral cavity via the parotid duct or Stensen duct (de Almeida, P Del Vigna, Gregio, Machado, De Lima, & Azevedo, 2008).

Submandibular or submaxillary glands are located beneath the lower jaws, superior to the digastric muscles (Kraehenbuhl & Neutra, 1992). Despite their small sizes, they secrete approximately 65-70% of saliva which is a mixture of both serous fluid and mucus (Pedersen, Schubert, Izutsu, Mersai, & Truelove, 1985). The saliva from submandibular gland enters the oral cavity via the submandibular duct or Wharton duct (Navazesh, 1993). This duct exit site is sublingual caruncle which is a pair of small prominence on either side of the frenulum under the tongue (Mandel, 1987).

Sublingual glands are positioned inferior to the tongue, anterior to the submandibular glands (Kraehenbuhl & Neutra, 1992). The secretion produced from sublingual glands is up to 5% of saliva with a mixture of serous and mucus but with more mucus concentration (Navazesh, 1993). Sublingual glands are connected into the mouth via eight to twenty excretory ducts called the Rivinus ducts (Catalán et al., 2012). Bartholin duct is the largest duct from Rivinus ducts that drains the saliva from the sublingual gland to submandibular duct into sublingual caruncle (de Almeida, P Del Vigna et al., 2008).

Each gland pours its secretions though specific ducts in the oral cavity. For SIg-A to be present inside these secretions and to commence any defense strategy, firstly, the antigen must be transported across the epithelial barrier to mucosal-associated lymphoid tissues (MALT) in the salivary glands, where the B cells and T helper cells are stationed (Woof & Kerr, 2006). The follicle-associated epithelium is the gateway for microfold cells (M cells) to deliver the antigen from the mouth inside the MALT areas. Secondly, the B cells interact with the antigen brought by M cells and respond by releasing SIg-A (Kraehenbuhl & Neutra, 1992). Finally, the SIg-A is actively transported by the epithelial polymeric Ig receptor on the basolateral surface of epithelial cells via transcytosis and endocytosis to be released into saliva through ducts (Mestecky & Mcghee, 1987).

One of the roles of the Slg-A is to be the major effector of human body's defense in the mucosal areas against microorganisms causing upper respiratory tract infection (URTI) which can be viral, bacterial, and fungal infections in the mouth, nose, sinuses, pharynx or larynx. (Filaire, Bonis, & Lac, 2004). By attaching to the pathogenic microorganisms, SIg-A inhibits or

prevents their entry into the body. The level of Slg-A is correlated with susceptibility or resistance to URTI (Mackinnon & Hooper, 1994). Low rates of SIg-A concentration might increase the risk of URTI between athletes (Gleeson, Hall, McDonald, Flanagan, & Clancy, 1999).

1.2 The Sympathetic Nervous System

The human body is constantly challenged. It needs to maintain homeostasis which is a state of balance within body systems. The autonomic nervous system is responsible for the involuntary homeostasis (Ekman, Levenson, & Friesen, 1983). The autonomic nervous system has two divisions which counteract each other to balance homeostasis: the sympathetic nervous system responsible for "fight or flight" reactions, and the parasympathetic nervous system responsible for "rest and digest" responses (Fulton, 1938). The activation of the sympathetic nervous system causes the secretion of epinephrine from the adrenal medulla and norepinephrine from sympathetic nerves (Radosevich et al., 1989). Epinephrine and norepinephrine act on the adrenergic receptors on the body's organs and tissues (Moore & Bloom, 1979).

The immune system is regulated by autonomic nervous system. The sympathetic nervous system has the capacity to regulate the immune system by secreting epinephrine and norepinephrine which is sensed by adrenergic receptors on immune cells (Kohm & Sanders, 2000; Takenaka et al., 2016). Fibers from the sympathetic nervous system also directly innervate the tissues in most immunological tissues, allowing the mediation of neuronal and hormonal responses (Felten, 1985).

1.3 The Sympathetic Nervous System and SIg-A

The sympathetic nervous system can be activated by a stressor (Elenkov, Wilder, Chrousos, & Vizi, 2000). A stressor is defined as a factor that challenges the homeostasis of the human body. The sympathetic nervous system reactions and activation of the hypothalamic-pituitary-axis typify some stress responses (Tsigos & Chrousos, 2002).

The sympathetic nerves are innervated to lymph nodes where white blood cells mostly communicate (Elenkov et al., 2000; Kohm & Sanders, 2000). The SIg-A concentrations elevate

in response to the sympathetic nervous system stimulus more than the parasympathetic nervous system in the submandibular gland inside rats (Carpenter, Garrett, Hartley, & Proctor, 1998). The alpha-adrenergic receptors, when blocked, downregulates the SIg-A regulations (Ring et al., 2000; Winzer et al., 1999). Facing an acute stressor, the sympathetic nervous system could increase the SIg-A concentrations but the elevated state is transient (Bristow, Hucklebridge, Clow, & Evans, 1997; Fan et al., 2009).

1.4 The CPT and SIg-A

Exposure to cold temperatures have been investigated as a stressor affecting the link between sympathetic stressors and immune changes (Ring et al., 1999; Ring et al., 2000; Willemsen et al., 1998; Winzer et al., 1999)

The cold pressor test (CPT) involves a brief immersion of a body limb hand, or foot, in cold water typically around 1-4˚ C between 30 seconds to 8 minutes. Cold exposure triggers a local vasodilation in the limb, and a central response which augments muscle sympathetic nerve activity and raises systolic blood pressure (SBP) and diastolic blood pressure (DBP) (Victor, Leimbach, Seals, Wallin, & Mark, 1987). The CPT is commonly used to study pain, hypertension, thermoregulatory mechanisms, and it can affect immune cells (Jiang, Morrow-Tesch, Beller, Levy, & Black, 1990). It is primarily a stimulator of the sympathetic nervous system resulting in a gradual rise in norepinephrine in the plasma, with little involvement of the parasympathetic nervous system (Atterhog, 1980).

The mechanism of action in CPT starts with the cold/pain sensing nerves in the cold-exposed skin. The skin has three layers; epidermis, dermis, and hypodermis (Khurana, 2006). Thermos-receptors, a subgroup of somatosensory nerves, and free nerve endings in the dermis layer of the skin covering all over the body oversee sensing temperature. Thermo-receptors are connected to sensory afferents of the sympathetic nervous system that can cause vasodilation or vasoconstriction (Fulton, 1938). The CPT leads to vasodilation in cold-exposed area and vasoconstriction in the other parts of the body which why the exposed area turns red due to blood overflow (Chwałczyńska, 2015).

The density of thermo-receptors changes in different parts of the skin in the body. As thermo-receptors in one area of the skin are denser, they are more sensitive in sensing the

ambient temperature (Seals, 1990). The sensitivity of these nerves from higher to lower ranges, respectively, belongs to the face, hand/ feet, and lastly the truck in the human body (Delves, Martin, Burton, & Roitt, 2016).

The CPT has also been used to investigate the SIg-A. However, studies investigating the CPT as a stressor resulted in controversial SIg-A reports; stating no change in SIg-A concentration, SIg-A secretion rate, and saliva volume (Winzer et al., 1999), a trend to increase SIg-A concentration but significant increase in SIg-A secretion rate and saliva volume (Willemsen et al., 1998), or a decrease in SIg-A concentration, SIg-A secretion rate, and saliva volume (Ring et al., 2000).

1.5 Salivary α-Amylase

Salivary α-amylase (SαA) is used as a surrogate biomarker for norepinephrine and an indicator of sympathetic activation with strong correlation to plasma norepinephrine secretion (Chatterton, Vogelsong, Lu, Ellman, & Hudgens, 1996). SαA is an enzyme that breaks starch-structured material inside the mouth (Ramasubbu, Paloth, Luo, Brayer, & Levine, 1996). SαA also plays a role in the immunity of the human body by binding to bacterial microorganisms such as the family of the oral streptococci bacteria (Scannapieco, Solomon, & Wadenya, 1994).

The sensitivity of the SαA to stress levels has caused this enzyme to be considered as a noninvasive biomarker for sympathetic nervous system activity (U. M. Nater & Rohleder, 2009). The SαA levels increases after psychological and physical stressors (U. M. Nater, 2004; Walsh, 1999).

The CPT, as the sympathetic nervous system stressor, induces elevated levels of SαA (van Stegeren, Wolf, & Kindt, 2008; Youssef et al., 2018). Considering the immune role of this abundant enzyme on one hand and the stress-induced sensitivity on the other, in this study we chose the SαA as a saliva biomarker to confirm the effect of our CPT procedure on the sympathetic nervous system activation of the participants.

1.6 The CPT and Cardiovascular and Hemodynamic Reactions

The sympathetic nervous system regulates cardiovascular reactions (Hines & Brown, 1936). In this study, we measured the heart rate (HR) in beat per minute (bpm) and hemodynamics of the heart in response to the CPT. The hemodynamics of the heart consists of; blood pressure (BP), stroke volume (SV), cardiac output (CO), pre-ejection period (PEP), left ventricular ejection time (LVET), mean arterial pressure (MAP), and total peripheral resistance (TPR).

The BP is the measurement of the pressure that the circulating blood enforces on the walls of the vessels, which has two divisions; systolic blood pressure (SBP) and diastolic blood pressure (DBP) (Hines, 1940). The SBP is the pressure exerted on the arterial walls and other vessels when in the active contraction phase of the ventricles and the DBP is the pressure exerted on the arterial walls when the heart is at rest between the beats (Thacker, 1940). The SV is the amount of blood that a ventricle pumps in one beat. The CO is the amount of blood that a ventricle pumps in one minute, the amount is measured by HR × SV (Cuddy, Smulyan, Keighley, Markason, & Eich, 1966). The PEP is the time it takes the heart to start the blood ejection into the aorta from the ventricular contraction to the opening of the valves (Mishra, Manjareeka, & Mishra, 2012). The LVET is the time it takes the heart to pump blood through the left ventricle into the aorta from the valve opening to the valve closing. The MAP is the mean pressure on the arterial walls from the beginning of one heartbeat to the beginning of the next which is total is a cardiac cycle (Cuddy et al., 1966). The TPR is the total amount of pulmonary vasculature resistance that heart must overcome to create blood flow (Wood, Sheps, Elveback, & Schirger, 1984).

The CPT has been widely studied in the past for the effect it has on HR and BP since it is a classic sympathetic stressor. Hines and Brown, who created and first implemented the CPT in research, used the CPT to classify people based on their BP reactions to hyper and hypo-responders, based on the change in SBP of 22 (mmHg) during the CPT (Hines, 1940).

2.0 Rationale and Objective

2.1 Rational

The purpose of this study was to determine if the CPT as a sympathetic stimulus causes an increase in SIg-A concentrations. The secondary purpose was to determine if the SIg-A changes caused by the CPT correlates with the changes in SαA concentrations and cardiovascular and hemodynamic parameters indicative of sympathetic activity. This study investigates the effect of the CPT on the amount of SIg-A proceeded in the saliva. The expected results will teach us more about the connection between the sympathetic nervous system activation and the SIg-A responses.

To our knowledge, there has not yet been a study that correlated SIg-A and SαA. It is not known if the magnitude of changes in SIg-A is correlated with the changes in SαA levels. The studies that have used the CPT to measure the effect on SIg-A or SαA have not measured both factors there is no data on SIg-A and SαA correlation after the CPT. The research investigating the SIg-A and cardiovascular and hemodynamic parameters changes by the CPT have been inconclusive.

Conducting research in a natural environment requires a non-invasive method to measure the sympathetic activation. The non-invasive nature of salivary measures allows us to investigate the individual differences in the sympathetic nervous system response in the laboratory as well as in a naturalistic setting. Understanding the SIg-A release into the saliva responses to the CPT could potentially be used to classify the immune system's responsiveness to the sympathetic nervous system into categories as enhancive or suppressive. Low rates of the SIg-A bring about the risk of URTI in athletes with high intensity exercise programs such as marathon runners (Nieman, 2007). By researching the pattern of response of SIg-A to stressors such as the CPT, it might be possible to predict and furthermore enhance the concentrations of SIg-A in people who are at risk of being affected by URTI.

2.2 Objective

The first objective is to provoke the sympathetic nervous system by the CPT and measure SIg-A, SαA, HR, and BP. The second objective is to investigate the relation between the SIg-A changes caused by the CPT with the changes in SαA concentrations and cardiovascular and hemodynamic parameters indicative of sympathetic activity.

2.3 Hypotheses

Hypothesis 1

The SIg-A concentrations will significantly increase during 5 minutes CPT and will return to baseline levels 5 minutes post CPT.

Hypothesis 2

The SIg-A changes induced by the CPT will correlate to the sympathetic-mediated SαA and cardiovascular parameters changes.

2.4 Aims

Aim 1

Perform the CPT on healthy participants (male and female) and collect saliva and cardiovascular hemodynamics using Suntech®Tango+ at 7 time-points, Nexfin®, and HIC-4000I® impedance cardiograph continuous monitoring.

Aim 2

Measure the levels of the SIg-A using an ELISA kit, and SαA using an α amylase kinetic reaction kit pre, during, and post the CPT.

Aim 3

Use Pearson's rank correlation coefficient to determine statistical dependence between the SIg-A changes induced by the CPT and the changes of other measures during and post the CPT.

3.0 Methods

3.1. Participants

The Concordia University Human Research Ethics Committee (certificate number: 30004539) approved of this study. Thirty healthy participants were initially recruited. Samples from 20 of these participants (7 males, and 13 females) were available and used due to having complete sample sets for this sub-study. The mean age of the 20 participants was 24.7 years (± 4.5), their mean weight 71.8 kg (± 13.9), and their mean height 169.7 cm (±7.9). All participants signed an informed consent. The recruitment was preceded by social media, e-mail, and a flyer. Health status was determined by self-reporting on a questionnaire. Exclusion factors included being affected by cardiovascular disorders, neurological disorders, diabetes, epilepsy, known hypertension, Raynaud's syndrome, pregnancy, smoking, the use of recreational drugs, and the use of medications that alter cardiovascular function.

The menstrual cycle was self-reported by the female prior to their participation, in order to maintain consistency among and to avoid confounding effects of varying circulating hormones. The participation schedule of the females was set during the follicular phase of their menstrual cycle (Stickford, VanGundy, Levine, & Fu, 2015), maximum up to seven days after the cessation of their last period.

For at least 12 hours prior to the study, the participants were instructed to avoid caffeine, exercise, and alcohol, and to avoid eating for at least 2 hours prior to the test. All participants signed a consent form before conducting the experiment. Participants were compensated with $15.

3.2 The Procedure

The experiments were conducted at Concordia University PERFORM Centre. The duration of each session was approximately 1.5 hours. Signing the consent form by the participant and explaining the study procedure was the first course of action. In the next step, they were asked questions about their age, sex, the duration of time before their last food consumption, their transportation to the PERFORM Centre, and whether they followed all the

pre-participation instructions. Female participants were asked to confirm that they had their last period within the last 7 days time frame. Weight and height were recorded.

Three devices were set up on the participants: the Suntech®Tango+, Nexfin®, and the HIC-4000I® impedance cardiograph. Participants sat quietly for at least 15 mins before actual recording of data. The experiment in total took 32 minutes to conduct for each participant (Figure 1). Within 15 minutes, three baseline saliva samples and were obtained at 5 minutes intervals, after which the water bath was brought into the room. Two minutes were allotted for positioning the water bath. These two minutes were excluded from analysis due to anticipatory responses are known to occur just before the CPT (Lacey & Lacey, 1962). A thermal cooler was used as the water bath which has affixed with freezer packs with a thermometer attached to the interior. The participants were instructed to submerge their right hand into the water bath about up to wrist level. The hand submerging took place for 5 minutes.

The saliva sampling was done as indicated in Figure 1. Cardiovascular and hemodynamic values such as SBP, DBP and HR measures were recorded continuously throughout the whole experiment by using the COP_WIN™ software. A towel was made ready for the participant's hand when removed from the cold water. The participants were allowed to have the towel wrap around their hand during the two final recordings in the last 10 minutes.

Regarding the water bath temperature, it remained at 3.1 ˚C (± 0.9˚C). All the experiments were conducted in the same closed room with a fixed room temperature of 21-22˚C.In order to minimize environmental factors, the same two researchers ran the tests.

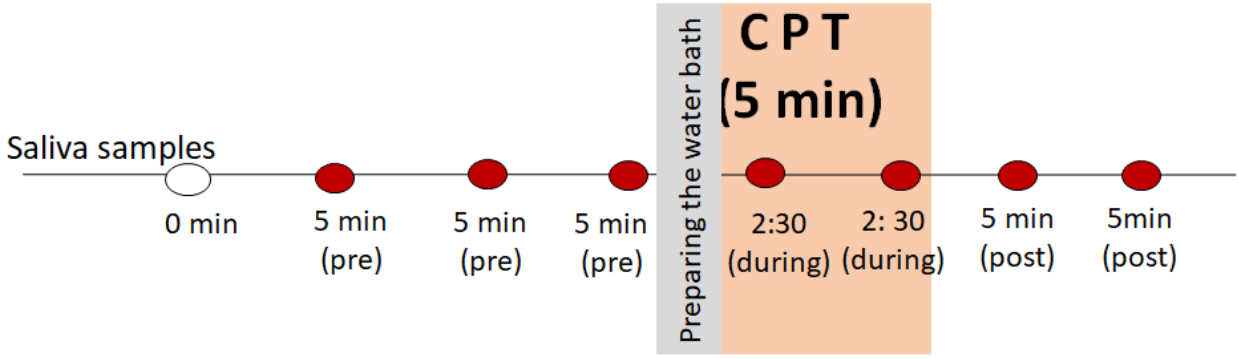


Figure 1. Schematic of Study Procedure.

Figure 1. The graph shows the timing of each saliva sampling. No sample was collected at 0 min (open circle). Samples were collected at the indicated intervals (filled circles). Three pre-test samples, two samples during the CPT, and two post-test samples were taken as shown. Two minutes were needed between the third pre-test and start of the test to bring the cold bath into the room are shown in a grey shaded area. The orange shaded area denotes the CPT when the hand was submerged.

3.3 Cardiovascular Measurements

To measure DBP, SBP, and HR, an automated blood pressure monitor (SunTech® Tango+) was attached to the right upper arm. This measurement was initiated manually 30 s before starting the CPT. A continuous hemodynamic monitoring system (Nexfin® beat-to-beat) was attached to the left finger and wrist to measure finger arterial pressure and HR in a continuous waveform.

To continuously monitor SBP, DBP, HR, and record the MAP, CO, and SV the Nexfin® beat-to-beat hemodynamic monitoring system was utilized. All the Nexfin® values had averages of 30sec. The settings of the Nexfin® utilization was according to the procedure-operating document "Use and Cleaning of Nexfin".

To record the continuous cardio dynamic activity, impedance cardiography (ICG) with electrode bands in full band configuration was used (Bacon et al, 2010; Sherwood et al., 1990).

The HIC-4000I® impedance cardiograph recorded The ICG signal, in coordination to the COP_WIN/HRV™ software (Bio-Impedance Technology, Inc., Chapel Hill, NC).

To continuously record HR, CO, SV, PEP, LVET, and PEP/LVET, the impedance cardiograph was utilized. The set-up of this device was according to the procedure-operating document "Use and Cleaning of HIC-4000I impedance cardiograph". 4 disposable mylar band electrodes (T8001 Bio-Impedance Technology, Inc., Chapel Hill, NC) picked up the signals. Around the base of the neck and the thorax at the level of the xiphoid process was the location of the first "recording" electrode. And at least 3cm below and parallel to the "recording electrodes" was marked for the "current" electrodes placement. The COP_WIN/HRV™ software averaged the distance between the two "recording" electrodes in cm in the front (parallel to the sternum) and the back (parallel to the spine). The data was continuously recorded at 60 Hz filter in 30 second ensemble averages (EA).

3.4 Cold Pressor Test

An ice-cold water bath monitored by a thermometer was used for the CPT. The vessel for the water bath was a cooler. To maintain the temperature at 4°C, ice packs were adjusted to cover all walls of the (except the side adjacent to the participant) and bottom of the cooler. The mean

water temperature for all participants was 3.1 ˚C (± 0.9˚C). All participants submerged their right hand to about wrist level for 5 minutes. Withdrawal of the hand from the cooler was considered as an exclusion from the study results. Two participants from the original study withdrew their hand due to pain but they were not included in this substudy because the data was incomplete.

3.5 Saliva Measurements

Participants were instructed to pool their saliva between their tongue and pallet and refrain from actively salivating, chewing or swallowing, to prevent the stimulation of the SαA secretion. Saliva was collected at the indicated seven time-points. The first three values (baseline values) and the final three values were averaged in analyzing the data.

A sealed sterile transfer pipette was inserted between the tongue and palette collecting the accumulated saliva. Roughly 0.5ml of the saliva sample from each participant were transferred and stored in 1.5ml micro-centrifuge tubes. Total saliva was weighed, and then divided into 20µl labeled aliquots before being frozen at -20°C until needed for analysis. Prior to analyzing the samples, they were thawed and centrifuged at 1500x g for 15 minutes. Saliva volume was estimated by weighing the vial immediately after collection with the assumption of saliva density being 1.00 g·ml-1 (Cole & Eastoe, 2014).

3.6 SαA

An α-amylase kinetic reaction kit (1-1902, (Single) 96-Well Kit, Salimetrics Inc, Carlsbad, CA) was used for samples' SαA analyze. The manufacturer's protocol was followed. In summary, a 1:10 dilution was prepared by mixing 10µl of each saliva sample with 90µl of diluent. The sample was then diluted to a 1:200 dilution by mixing the addition 10µl of the 1:10 to 190µl of diluent. High and low salivary α-amylase 8µl of the pre-diluted controls and the 1:200 saliva dilutions were plated onto a 96-well plate. Samples were plated in duplicate. 300µl of α-amylase substrate pre-heated at 37°C was added to the plate, one row at a time and was read through a 405nm filter. Readings were taken at 1 and 3 minutes, time point 3 was subtracted from time point 1 and multiplied by 328 to get the activity measure of salivary α-amylase in Units per milliliters (U/ml).

3.7 SIg-A

Salivary Ig-A was measured using a SIg-A ELISA kit (Salimetrics Inc., cat 1-1602, Carlsbad, CA). Firstly, 25 µL of saliva into 100 µL of 1X SIg-A diluent to 1:5 dilution. Then, the sample plate was prepared by adding 4 mL of 1X SIg-A diluent to each tube and adding 10 µL of the standard (diluted according to Reagent Preparation), control, or diluted unknown saliva sample. The zero tube was filled with 10 µL of 1X SIg-A diluent.

The antibody-enzyme conjugate 1:120 was diluted by adding 25 µL of the conjugate to the 3 mL of 1X SIg-A diluent and 50 µL of the diluted antibody-enzyme was conjugated to all tubes. The samples were incubated for 90 minutes at room temperature. 50 µL from each tube sample was pipetted to the microtitre plate and covered with the adhesive plate sealer which was already provided in the kit. The tubes, then, were incubated at room temperature with continuous mixing at 400 rpm for 90 minutes. The plate was washed 6 times with 1X wash buffer and was blotted on paper towels before turning upright. 50 µL of TMB solution was pipetted to each well which afterward was mixed on a plate rotator for 5 minutes at 500 rpm. The plate was incubated in the dark at room temperature for an additional 40 minutes. By adding 50 µL of stop solution the last stage was done. The plate was put on a plate rotator for 3 minutes at 500 rpm to mix the stop solution thoroughly, which turned the wells to yellow. Lastly, after wiping off the bottom of the plate, it was put in a plate reader for measuring absorbance at 450 nm.

3.8 Data Analysis

The Suntech® Tango^{+} cuff collected blood pressure and HR measures from the blood pressure at the 7 different time points were averaged for the 20 participants. This resulted in the average SBP, DBP, and HR values.

The Nexfin® provided values for the 32 minutes whole span of the study in every 30 seconds time. The raw data was transferred into an excel file and the data was averaged resulting in a value that was representative of each of the 7 time-points.

3.9 Statistical Analyses

3.9.1 T-test

For comparing all baseline measures between male and females an unpaired two-tailed t-test was used using SPSS (IBM statistics 24 software for Windows). A 95% confidence interval was used for all t-tests; they were all computed using SPSS and the data were manually put on the table.

3.9.2 Repeated Measures ANOVA

A repeated measure One-way ANOVA was used to determine the significant difference in SIg-A, SαA levels, cardiovascular and hemodynamic parameters measured at three different periods; baseline, during CPT and post CPT. A p-value <0.05 was used for the analysis (GraphPad Prism, version 5.01 for Windows, Graphpad Software, Inc. 2007). Tukey's post hoc test was used to investigate the between groups variety as it determines which of the three groups were different from each of the other groups.

3.9.3 Calculating Delta Values (Δ1 and Δ2)

Once baseline, during CPT, and post CPT averages were computed for SIg-A, SαA levels, and all hemodynamic and cardiovascular parameters, Δ1 and Δ2 were calculated.

Δ1 = During CPT – baseline values.

Δ2 = Post CPT – baseline values.

Deltas were used to determine the magnitude of change in each parameter. All delta calculations were done on SPSS (IBM statistics 24 software for windows).

3.9.4 Pearson's Correlation Coefficient

Two-tailed Pearson's correlation coefficient was used to measure statistical dependence between two variables. SIg-A, SαA levels at baseline, CPT, recovery, in addition to the delta for SIg-A, SαA levels were correlated with cardiovascular and hemodynamic parameters that reflect

sympathetic activity. A 95% confidence interval was used. SPSS was used (IBM statistics 24 software for windows) to compute the correlations.

4.0 Results

4.1 Baseline Parameters

The SIg-A was measured from a total of twenty healthy participants, 13 women with and 7 men, who completed a 5-minute CPT with continuous hemodynamic and cardiac measurements. The average mean age of the participants was 24.7 years (± 4.5), their mean height 169.7 cm (±7.9), and their mean weight 71.8 kg (± 13.9). The baseline parameters between men and women were compared using an unpaired two-tailed student's t-test and a 95% confidence interval. Male participants were heavier, taller, and older as compared to female participants. Male participants had higher SBP and DBP baseline values and females had higher HR baseline values (Table 1).

Table 1. Physical Characteristics of Participants

Variables	**Total Mean N= 20**	**Male N=** 7	**Female N=** 13
Age (years)	24.7 ±4.5	28.57 ± 4.7 **	22.6 ± 2.7
Height (cm)	169.7 ±7.9	177.5 ±3.7 ***	165.5 ± 6.09
Weight (kg)	71.8 ±13.9	83.08 ± 8.6 **	65.7 ± 12.5
SBP (mmHg)	117.1 (14.4)	129.1 (7.6) **	110.6 (13.02)
DBP (mmHg)	70.8 (8.8)	78.4 (8.5) **	66.7 (6)
HR (Bpm)	71.5 10.8)	63.5 (10.5)	75.8 (8.4) *

*Table 1. *p < 0.05, ** p < 0.01, *** p < 0.001 p-value for un-paired two-tailed t-test between men and women.*

4.2 Effect of CPT on SIg-A

There was a considerable range of baseline values of SIg-A, and the pattern of response varied for each person. The range of baseline measures was between 14 and 136 (µg/mL). TDuring CPT, the SIgA in some people went down, others had no change, and some people their SIg-A went up. The range of changes observed was -52.8 ≤ +63.8 (µg/mL), where minus signifies a decrease, the plus signifies an increase in SIg-A. When the data were averaged for all subjects, the SIg-A levels had no significant change during CPT and continued the same pattern in post CPT (Figure 2). None of the time-points showed a significant change compared to the other time-points ($p > 0.05$). The trend of the mean SIg-A baseline was 53.3 (µg/mL) which increased by 0.2 (µg/mL) during the CPT phase and decreased in the post CPT phase to 53.4 (µg/mL) without having a statistical significance. There was no significant difference between women and men SIg-A levels ($P > 0.05$).

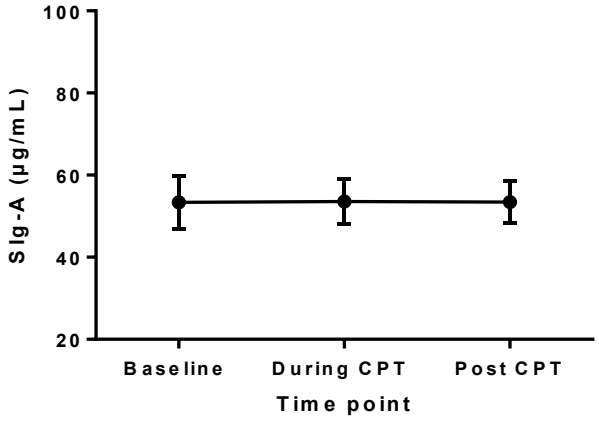


Figure 2. SIg-A Levels During and Post CPT

The graph shows the SIg-A data. A repeated measure one way ANOVA with $p<0.05$ was used to determine differences between the 3 time-points and a post hoc Tukey's Test was used to determine the difference between the mean of each group compared to the other two groups. Bars represent SEM.

4.3 Effect of CPT on SαA

The CPT induced an increase in SαA levels ($P < 0.01$) (Figure 3). During CPT levels of SαA were only 19.6 U/mL higher than baseline, which is likely the reason for no calculated statistical difference. However, when post CPT values were compared to baseline, there was a difference ($p < 0.5$). Levels of SαA were higher at post CPT (171.1 U/mL) than at baseline (120.8 U/mL) by 50.3 U/mL ($p<.05$) (Table 2).

When the during CPT values were compared to post CPT values, there was a difference, with post CPT values being 30.7 U/mL higher than during CPT values ($p<.05$). The values did not return to baseline in the post CPT period. The changes in SαA experienced by men and women throughout the study were compared using an unpaired two-tailed student's t-test and a 95% confidence interval. No difference between these two groups based on sex was observed at any of the 3 time-points.

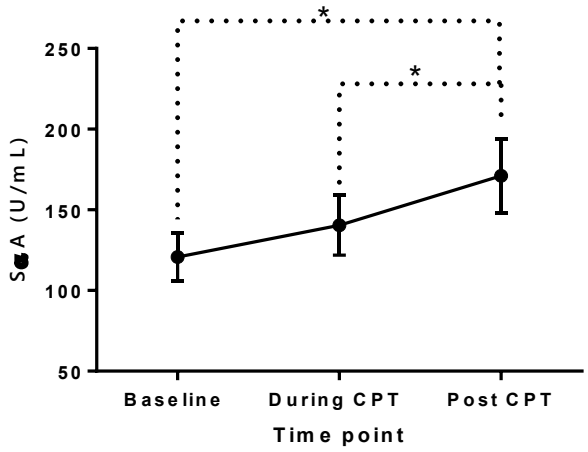


Figure 3. SαA Increases During and Post CPT

*SαA data are shown in this graph. A repeated measure one way ANOVA with $p<0.05$ was used to determine differences between the 3-time points and a post hoc Tukey's Test was used to determine the difference between the mean of each group compared to the other two groups. Bars represent SEM. * $p < 0.05$.*

4.4 Effect of CPT on Saliva Volume

Mean saliva volume was measured at the three time-points (Figure 4). The repeated measures ANOVA showed significant changes during the three time-points in total, ($P < 0.05$).

The comparison between during CPT and post CPT by a post hoc Tukey's Test showed there was a significant increase ($P < 0.05$). There was no other significance between the time points.

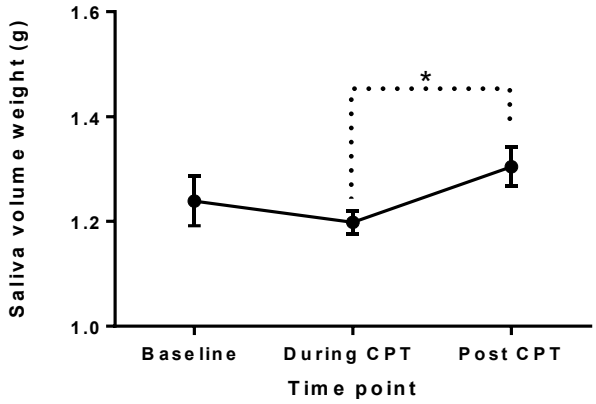


Figure 4. Saliva Volume Changes Due to the CPT

*Saliva data are shown in this graph. A repeated measure one way ANOVA with $p<0.05$ was used to determine differences between the 3 time-points and a post hoc Tukey's Test was used to determine the difference between the mean of each group compared to the other two groups. Bars represent SEM. * $p < 0.05$.*

4.5 Effect of CPT on HR

The CPT induced an increase in HR levels ($P < 0.01$) (Figure 5). Baseline HR rate compared to during CPT phase had slightly higher rates, there was no significance in the HR change during CPT by having only 2.9 bpm higher than baseline. On the other hand, mean HR during CPT phase (74.3 bpm) compared to post CPT (68.9 bpm) was significantly lower, there was a difference ($p<.001$), with post CPT values being (5.4 bpm) lower than during CPT values.

When HR in the post CPT values was compared to those of the baseline, there was a difference ($p<.05$). HR rate was lower at post CPT phase (68.9 bpm) than at baseline (71.4 bpm) by -2.4 ($p<.001$) (Table 2).

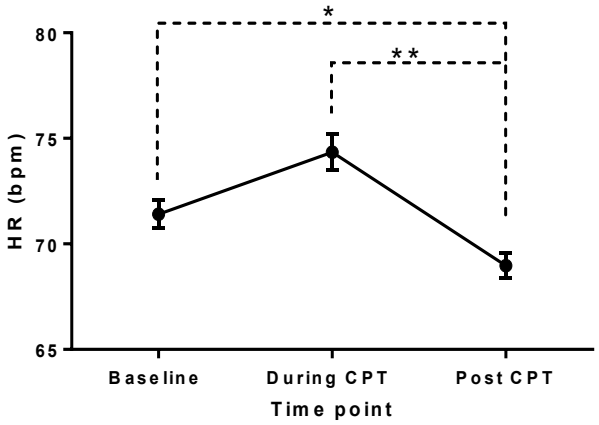


Figure 5. HR Changes Due to the CPT

*HR data are shown in this graph. A repeated measure one way ANOVA with $p<0.05$ was used to determine differences between the 3 time-points and a post hoc Tukey's Test was used to determine the difference between the mean of each group compared to the other two groups. Bars represent SD. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

4.6 Effect of CPT on Cardiovascular and Hemodynamic Parameters

The SBP ($p < 0.0001$), and DBP ($p < 0.0001$) were increased due to the CPT. The CPT caused elevated levels of the cardiovascular and hemodynamic measures influenced by changes in blood pressure and sympathetic activation: MAP ($p < 0.0001$), TPR ($p < 0.0001$), and LVET ($p < 0.0001$) (Figure 6-7, Table 2).

Both the SBP and DBP at post CPT values were higher than baseline ($p < 0.0001$ and $p<0.001$). The cardiovascular and hemodynamic parameters MAP, LVET, and calculated TPR (TPR=MAP/CO) remained elevated post CPT phase when compared to baseline. PEP remained slightly below baseline levels at post CPT. Consequently, so did PEP/LVET significantly ($p<0.0001$).

The sympathetic nervous system's activation through cold exposure, PEP a cardiac marker of sympathetic activation decreased ($p < 0.01$), resulting in a decrease in PEP/LVET ($p < 0.001$) during and post CPT when compared to baseline. There was an increase in CO levels ($P < 0.5$) which returned to baseline levels at post CPT. The SV had a trend to increase post CPT when compared to during CPT but it was not significant ($p > 0.05$), (Figure 7, Table 2).

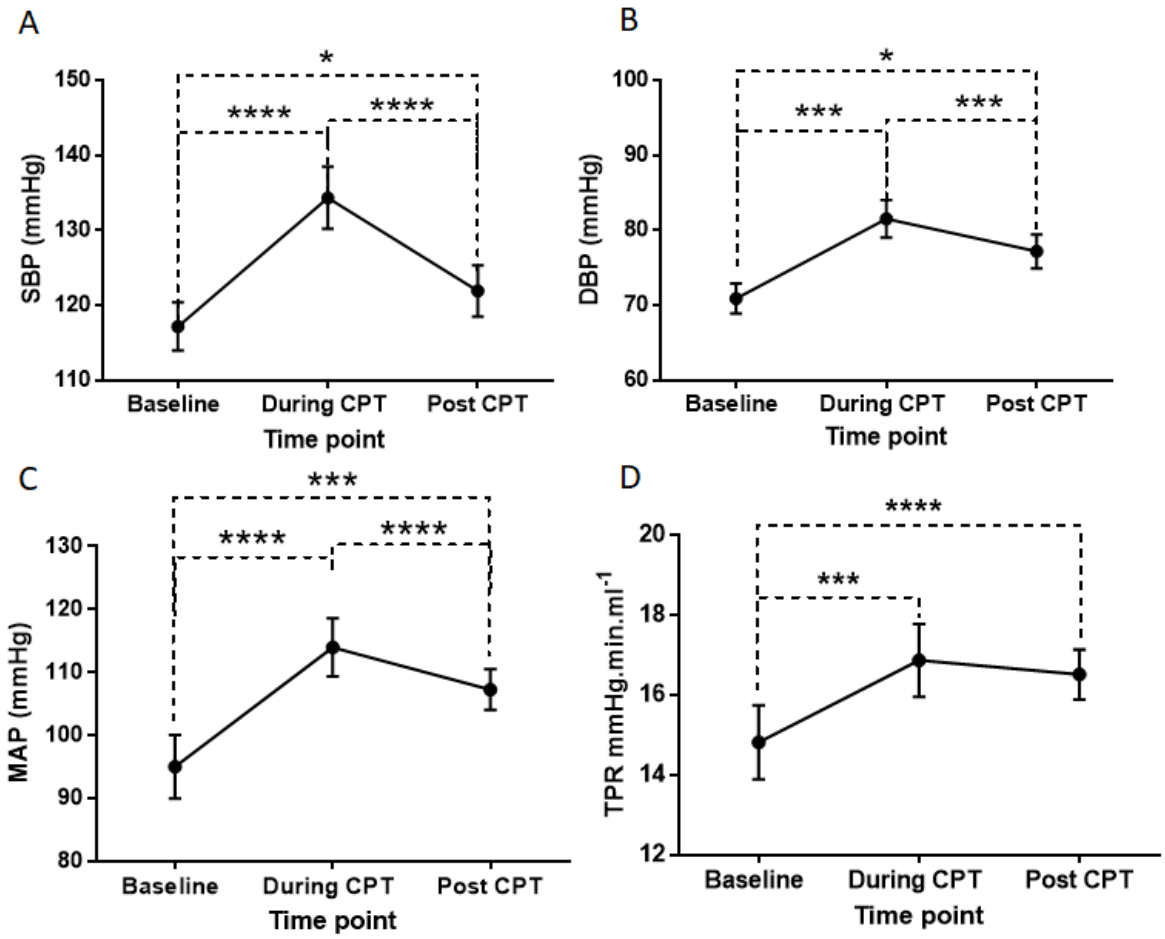
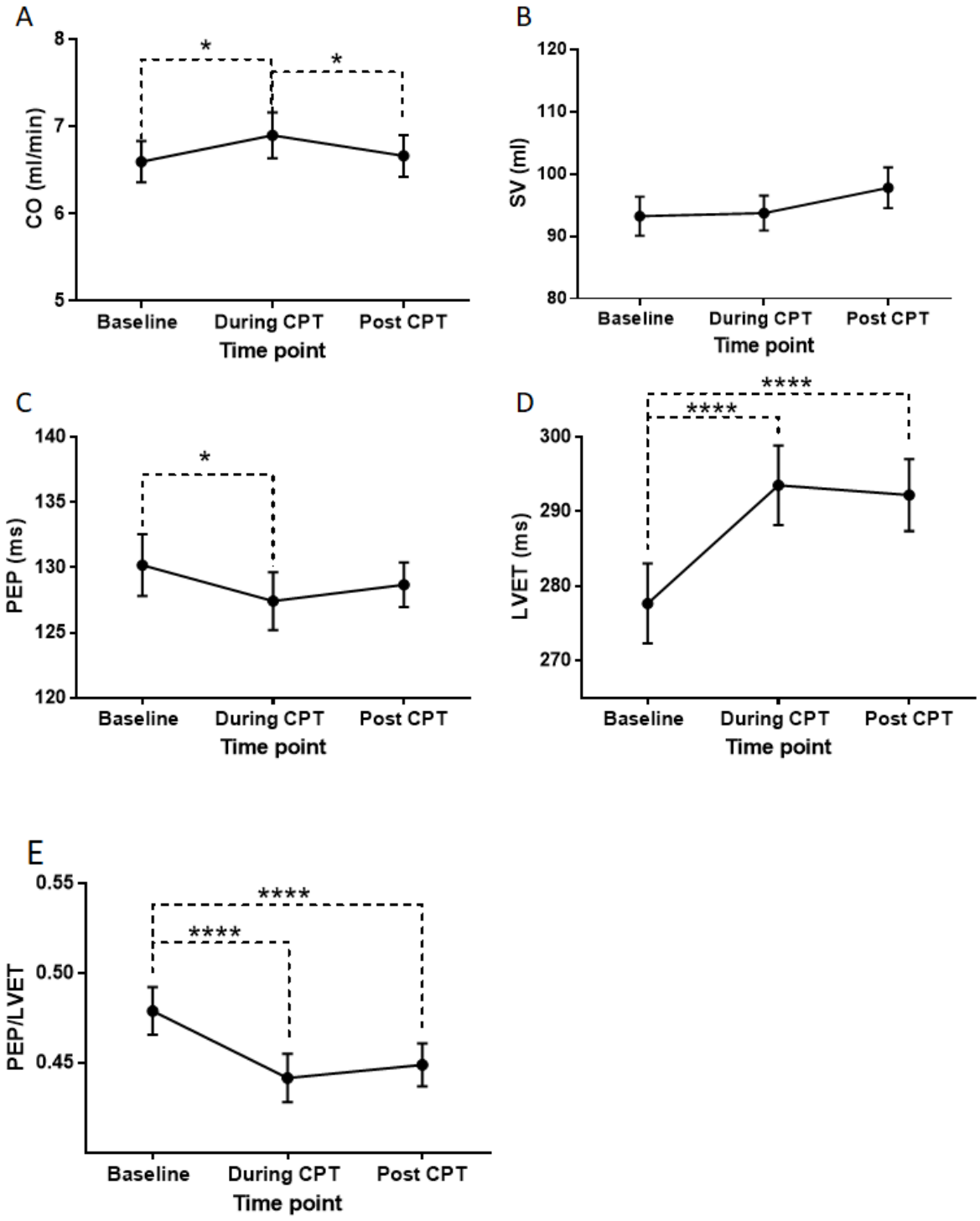


Figure 6. Hemodynamic Changes Due to the CPT

*A) SBP, B) DBP, C) MAP, and D) TPR data are shown in this graph. A repeated measure one way ANOVA with $p<0.05$ was used to determine differences between the 3 time-points and a post hoc Tukey's Test was used to determine the difference between the mean of each group compared to the other two groups. Bars represent SEM. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.*


A
CO (ml/min)
8
7
6
5
*
*
Baseline
During CPT
Post CPT
Time point
B
SV (ml)
120
110
100
90
80
Baseline
During CPT
Post CPT
Time point
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PEP (ms)
140
135
130
125
120
*
Baseline
During CPT
Post CPT
Time point
D
LVET (ms)
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Post CPT
Time point
E
PEP/LVET
0.55
0.50
0.45
****
****
Baseline
During CPT
Post CPT
Time point

Figure 7. Additional Hemodynamic Changes Due to the CPT

*A) CO, B) SV, C) PEP, D) LVET, and E) PEP/LVET data are shown in this graph. A repeated measure one way ANOVA with $p<0.05$ was used to determine differences between the 3 time-points and a post hoc Tukey's Test was used to determine the difference between the mean of each group compared to the other two groups. Bars represent SEM. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$*

Table 2. Mean of Saliva, Cardiovascular, and Hemodynamic Parameters

Variables	Baseline	During CPT	Post CPT
SIg-A (µg/mL)	53.3 ±28.5	53.5 ±24.6	53.4 ±22.8
SαA (Units/ml)	120.8 ±67.1	140.4±82.7 ≠	171.1±102.2 §
SBP (mmHg)	117.1±14.4 ****	134.3±18.5 ###	121.9±15.3 §
DBP (mmHg)	70.8 ±8.9 ***	81.5 ±11.1##	77.1 ±10 §
HR (bpm)	71.4 ±9.8	74.3± 11.1 ≠	68.9 ±10.4 §§
MAP (mmHg)	95.1 ±7.4 ****	114 ±11.1 ###	104.7±9.7 §§§
TPR (mmHg•min•ml^{-1})	14.8 (2.8) ***	17.02 ±3.4	16.1 ±§§§§
CO (ml/min)	6.5 ±1.03*	6.9 ±1.1≠	6.6 ±1.04
SV (ml)	93.2 ±13.6	93.7 ±12.2	97.7 ± 14.2
PEP (ms)	131.7 ±13.6 *	128.8±12.2	129.9 ±12.1
LVET (ms)	278.01±21.9 ****	293.2±24.1	293.08 ±25.6§§§§
PEP/LVET	0.47±0.06 ****	0.44 ±0.05	0.44 ±0.06§§§§

*Table 2. Saliva, cardiovascular, and hemodynamic parameters measured during the three time-points were averaged during baseline, during CPT, and post CPT. Each mean is reported with the relative standard deviation (SD). *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 for mean baseline vs. mean during CPT, ≠p < 0.05, ## p < 0.01, ### p < 0.001, #### p < 0.0001 for mean during CPT vs. mean post CPT, §p < 0.05, §§p < 0.01, §§§p < 0.001, §§§§p < 0.0001 mean baseline vs. mean post CPT. N= 20.*

4.7 Correlation Between the Range of Changes in SIg-A and Other Measures

The baseline, the changes during the CPT procedure (Δ1), and the changes induced post the CPT (Δ2) are the three values that we calculated for all our measurements (Table 3). We investigated the correlation between the named three values in the measures from saliva; SIg-A and SαA, cardiovascular system; SBP, DBP, HR, and PEP (Table 4).

The two-tailed Pearson's correlation between the measures at baseline SIg-A and baseline SαA and baseline SBP showed positive correlations ($P < 0.05$ and $r = 0.55$). The baseline DBP and Δ2 SIg-A correlated significantly ($P < 0.01$ and $r = 0.48$). There were no correlations between baseline, Δ1, and Δ2 SIg-A and the sympathetic measures such as Δ1 and Δ2 of SαA, SBP, DBP, HR, and PEP.

Table 3. Magnitude of Changes in Saliva, Cardiovascular, and Hemodynamic Parameters

Variables	Δ1	Δ2
SIg-A (µg/mL)	- 0.22 ±27.06	0.04 ±25
SαA (Units/ml)	19.6 ±74.3	50.3 ±70.4
SBP (mmHg)	17.1 ±11.8	4.7± 8
DBP (mmHg)	10.6 ±9	6.2 ±6.1
HR (bpm)	2.9 ±6.3	- 2.4 ±4
MAP (mmHg)	18.9± 9.1	9.6 ±5.7
TPR ($mmHg \bullet min \bullet ml^{-1}$)	2.2 ±1.6	1.3 ±1.1
CO (ml/min)	0.3 ±0.4	0.06 ±0.3
SV (ml)	0.51 ±4.9	4.57 ±4.5
PEP (ms)	- 2.8 ±4.3	- 1.7 ±3.5
LVET (ms)	15.2 ±9.4	15 ±8.4
PEP/LVET	- 0.03 ±0.2	-0.02 ±0.01

Table 3. The table shows the magnitude of changes in the measured parameters. The changes (Δ) in these parameters were calculated by subtracting. Δ1= (mean during CPT – mean baseline) and Δ2 = (mean post CPT– mean baseline).

Table 4. Baseline SIg-A and Δ SIg-A Correlations to Other Parameters

Variables	Base. SIg-A (µg/mL)	Δ1 SIg-A (µg/mL)	Δ2 SIg-A (µg/mL)
Base. SαA (Units/ml)	0.55 *	-0.36	- 0.2
Δ1 SαA(Units/ml)	- 0.35	0.18	- 0.14
Δ2 SαA (Units/ml)	- 0.06	- 0.12	- 0.37
Base SBP (mmHg)	0.55 *	0.39	0.37
Δ1 SBP (mmHg)	- 0.39	0.39	0.23
Δ2 SBP (mmHg)	0.30	0.30	0.15
Base. DBP (mmHg)	- 0.31	0.03	0.48 *
Δ1 DBP (mmHg)	- 0.20	0.24	0.08
Δ2 DBP (mmHg)	- 0.28	0.21	0.10
Base. HR (bpm)	0.07	- 0.18	- 0.33
Δ1 HR (bpm)	- 0.14	0.13	0.01
Δ2 HR (bpm)	0.02	- 0.36	0.04
Base. PEP (ms)	0.27	- 0.01	0.19
Δ1 PEP (ms)	- 0.20	0.002	0.07
Δ2 PEP (ms)	- 0.38	0.001	0.16

*The table shows the correlation of the SIg-A change during and post the CPT to the range of the changes in other measures during and post the CPT. Δ1= (mean during CPT – mean baseline) and Δ2 = (mean post CPT– mean baseline). Each mean is reported with the relative standard deviation (SD). Base = baseline, * $p < 0.05$.*

4.8 Correlation of Baseline SIg-A and Its Changes During CPT

As reported in section 4.2, there was a lot of variation in the baseline value and the change in SIg-A during CPT. We speculated that the baseline value of SIg-A when the person is resting would correlate to the magnitude change in SIg-A during CPT. Consistent with this idea, there was a significant, inverse correlation between the magnitude of SIg-A change (Δ) and baseline values ($r = -0.60$, $p = 0.01$), (figure 8). People with high baseline SIg-A had lower SIg-A during CPT, while people with low baseline SIg-A had higher SIg-A during CPT. People around the 50μg/mL mark in the baseline had very little change in SIg-A during CPT.

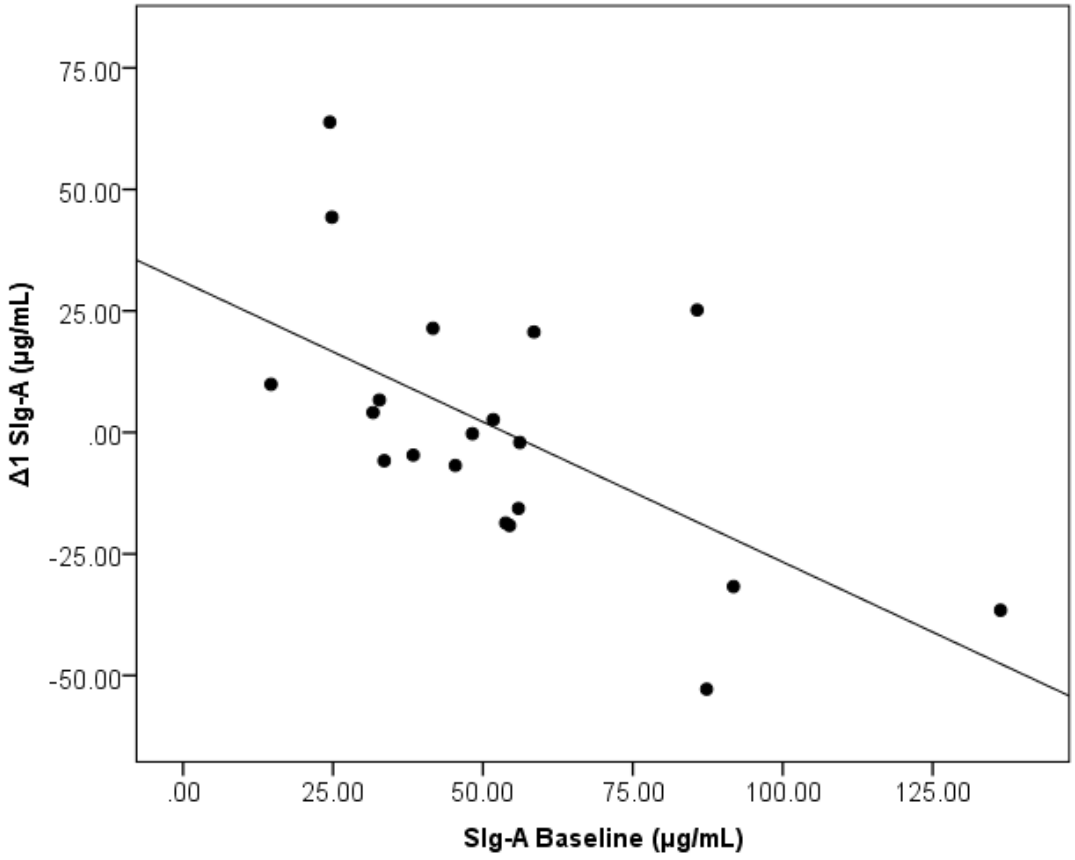

Figure 8. The Baseline SIg-A Correlation to Δ1 SIg-A.

The graph represents the SIg-A baseline values' correlation to the change in SIg-A during the CPT (Δ1 SIg-A).

Discussion

The purpose of this study was to determine if the CPT as a sympathetic stimulus causes an increase in SIg-A concentrations. The secondary purpose was to determine if the SIg-A changes caused by the CPT correlates with the changes in SαA concentrations and cardiovascular and hemodynamic parameters indicative of sympathetic activity. We performed the CPT on 20 healthy participants, 13 women and 7 men. The number of participants in the previous studies investigating the CPT suggests that our number of participants were in an acceptable range such as a study done by Winzer et al. (1999) with 20 participants, 9 women 11 men. Another study, Ring et al. (2000) conducted CPT on 27 participants, 20 women and 7 men. Willemsen et al. (1998) investigated the effect of the CPT on 16 men only. The uneven ratio of women and men in our study was due to this being a substudy. The original study had 30 participants 16 women and 14 men however there were not sufficient saliva samples from 10 people of the original study to be analyzed (Youssef, 2016).

There were differences in the cardiovascular system based on sex. Males had higher resting SBP and DBP than the female participants in this study which also have been reported previously (Kilgour & Carvalho, 1994). Resting HR in female participants was the only one baseline measure that was higher than the male participants. Which could be the reason for the higher resting SV in males when compared to females (SV= CO/HR). The baseline values for CO had no sex difference. The SIg-A and SαA baseline values also showed no difference between male and female participants.

Our primary hypothesis was to determine if the SIg-A levels will significantly increase in response to the CPT and will return to baseline levels in the period post CPT. We measured SIg-A, due to its important role as the immune system's first line of defense in the most exposed areas to the external environment such as the mouth, and its abundant concentrations compared to other antibodies, non-invasive collection, and low costs of analysis. The connection between the sympathetic nervous system and the SIg-A can be explained by the innervation of the sympathetic nerves to the lymph nodes (Elenkov et al., 2000; Kohm & Sanders, 2000). Stimulating the sympathetic nervous system and parasympathetic system in rats' salivary glands elevated the SIg-A concentrations (Carpenter et al., 1998). And the CPT affects the regulation of

the SIg-A concentrations through alpha-adrenergic receptors (Ring et al., 2000; Winzer et al., 1999).

Despite the diverse individual changes in SIg-A, there was no significance in the SIg-A concentration change during and post CPT. This data suggests the connection between the sympathetic activation and the SIg-A concentrations is indirect. This is consistent with a study showing no change in SIg-A to the CPT during an 8-min CPT (N= 20) on both hand in 10˚, 4-min each hand (Winzer et al. 1999). Also, Willemsen et al. (1998) observed only a slight increase in SIg-A concentration after the CPT (N= 16) for 4-min on one hand in 10˚.

Our investigation is not consistent with a paper by Ring et al. (2000) that conducted the CPT (N= 27) for 8-min on both hand in 10˚ (4-min each hand) which resulted in the significant reduction in SIg-A concentration. Other than the CPT, the reports concerning SIg-A's pattern of response to sympathetic stressors vary in physical and psychological stimuli. Bosch et al. (2001) observed significant decreasing levels of SIg-A concentration after watching a gory surgical video (N= 34) (Bosch et al., 2001), and Kugler et al. (1996) reported that one coaching session in a soccer match (N= 17) increases the SIg-A concentrations.

Our secondary hypothesis was to determine if the SIg-A changes induced by the CPT will correlate to the sympathetic-mediated SαA and cardiovascular parameters changes. The SIg-A response pattern surprisingly did not correlate to most of the sympathetic parameters except the baseline of SαA and SBP measures. The SIg-A concentration had no change while the sympathetic activation caused the SαA, hemodynamic parameters and cardiovascular measures to increase.

As expected, the SαA concentration increased during the CPT (Youssef, 2016) and reached its peak level in the post CPT phase. The actual peak time of SαA is not known since the data was not collected more than 10 minutes after the CPT. Our findings are consistent with Van Stegeren et al. (2008) investigation to measure the SαA in which they performed the CPT at less than 3˚ C on 80 participants for 3-min, they also performed a picture viewing along with rest and reading and reported a significant increase in the SαA.

In my study, the SBP and DBP increased profoundly which is consistent with previous studies (Hines & Brown, 1936; Hines, 1940; Mishra et al., 2012). The rise in systemic vascular resistance is most likely the cause of this augmentation (Cuddy et al., 1966), which also can explain the increase in TPR and MAP caused by the CPT. The pattern of changes in TPR and

MAP was similar to that of SBP and DBP. All the 4 parameters of SBP, DBP, MAP, and TPR peaked in the during CPT phase and decreased in the post CPT phase, but did not fall back to baseline levels. Apparently, 10 minutes of follow up measurement were not long enough for SBP, DBP, MAP and, TRP to go back to baseline levels. These findings suggest that, even after an acute disrupter of homeostasis such as the CPT, the sympathetic nervous system in the body requires more than 10 minutes, to re-establish resting states. PEP time was shorter, and LVET time was longer during the CPT suggesting the sympathetic signal to the heart muscle was elevated. The PEP values did not correlate to change in SIg-A, which argues against a link between sympathetic outflow and SIg-A levels.

This study demonstrates that immune responses can vary by individuals and are dependent on the baseline levels as the body tries to balance its state. The changes in the SIg-A concentrations after the CPT showed a robust negative correlation with its baseline. The immune response pattern observed in this study indicates that after exposure to cold stress, those with high baselines had a decrease in their values, conversely, those with low baselines had an increasing rate of SIg-A values, and those whom their baseline measures were at midline did not experience much change in SIg-A.

One aspect of stress response research that is often neglected is Wilder's law of initial value (Lacey & Lacey, 1962). The law of initial value was built upon empirical results from cardiovascular and immunological responses to autonomic drugs (Wilder, 1962). Wilder's research in the field of "basimetry", or measuring the baseline, has brought him upon the law of initial value. Wilder states in his book that:

"Given a standard stimulus and a standard period of time, the extent and direction of response of a physiological function at rest depends to a large measure on its initial (pre-experimental) level. The relations are as follows: the higher the initial value, the smaller the response to function-raising, the larger the response to function-depressing stimuli. Beyond a certain medium range of initial values there is a tendency to paradoxic (reversed) responses, increasing with extremeness of initial values." (Wilder, 1967).

One of the limitation of this study is the lack of saliva flow and SIg-A secretion rate measurements that has a potential of showing deeper understanding of SIg-A concetrations.

Mouth and oral cleaning such as a periodontal disease can alter the concentrations of the SIg-A (Marcotte & Lavoie, 1998b). Future studies can consider this as a cofactor.

This study did not follow the participants for URTI symptoms. As the future direction of this study, investigating the SIg-A pattern of response to the CPT as a sympathetic nervous system's stressor can add on to the knowledge of how this antibody functions which may help the future studies to predict the occurrence of the URTI.

In conclusion, this study suggests that the sympathetic stimulus has an indirect effect on the SIg-A response, following the law of initial value. Most people in the study had different baseline SIg-A, however, during the CPT the SIg-A changed differently in each person's saliva. These findings will help to interpret and analyze experiments concerning SIg-A measures as an indicator of immune responses mostly in athletes who exercise intensely such as marathon runners due to their low rates of SIg-A that puts them in risk of URTIs. This study might contribute to finding an approach towards preventing the occurrence of URTI in athletes after high intensity exercise.

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Appendix 1

In order to choose the agreeable statistical analysis, first, the data must undergo one of the categories of parametric or non-parametric. The first assumption of having parametric data is normal distribution. The Shapiro-Wilk test of normality on the SIg-A baseline data showed a significance of 0.05. Since the Sig value is over 0.05 the SIg-A data is normally distributed. Also, the Normal Q-Q Plot showed that the data distribution has a linear pattern.

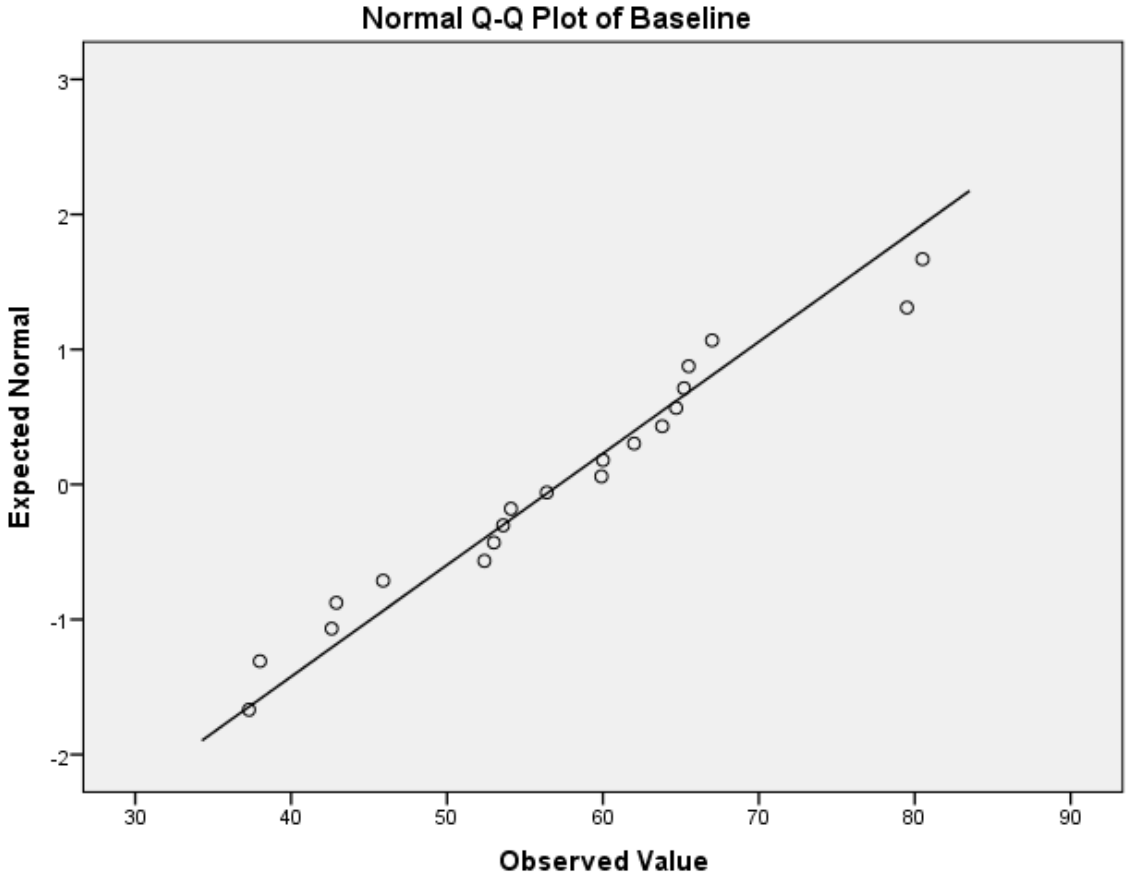
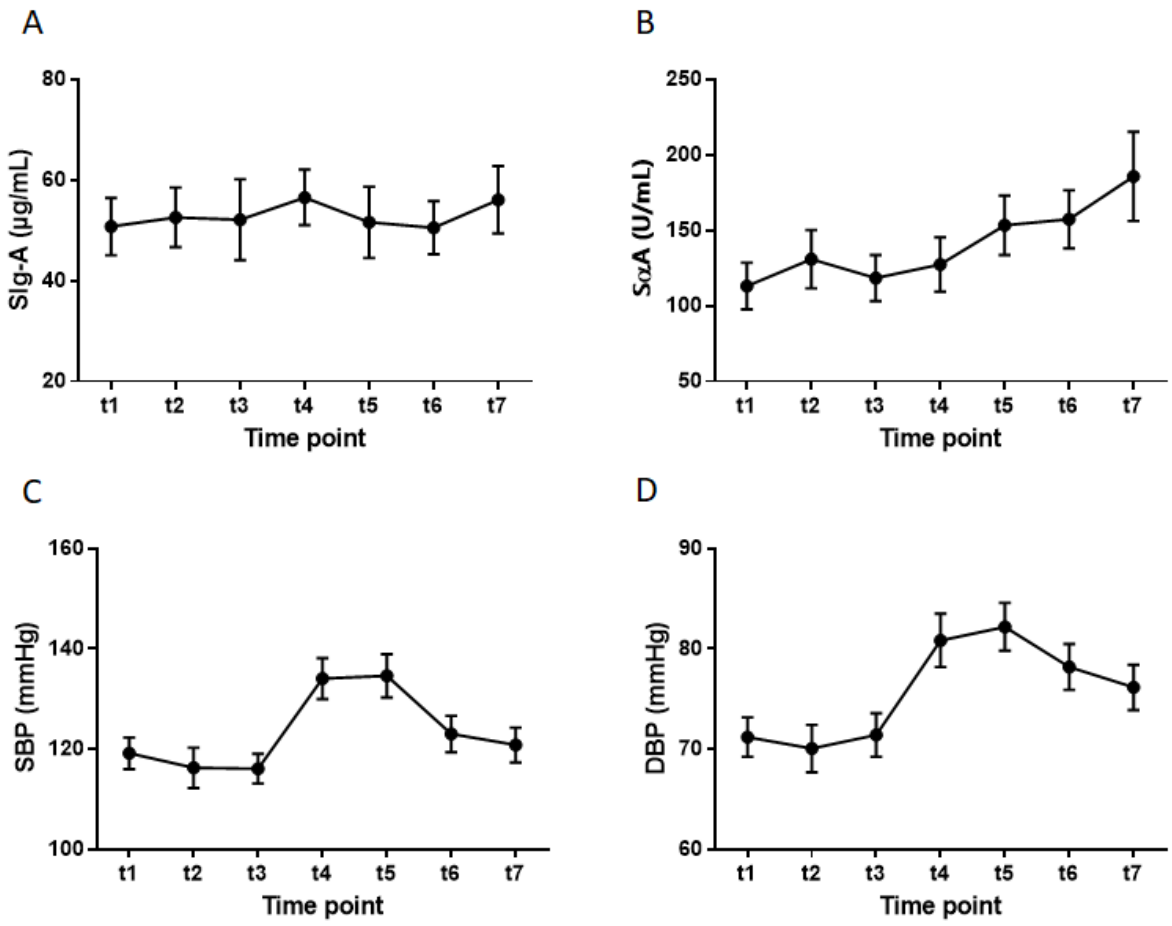


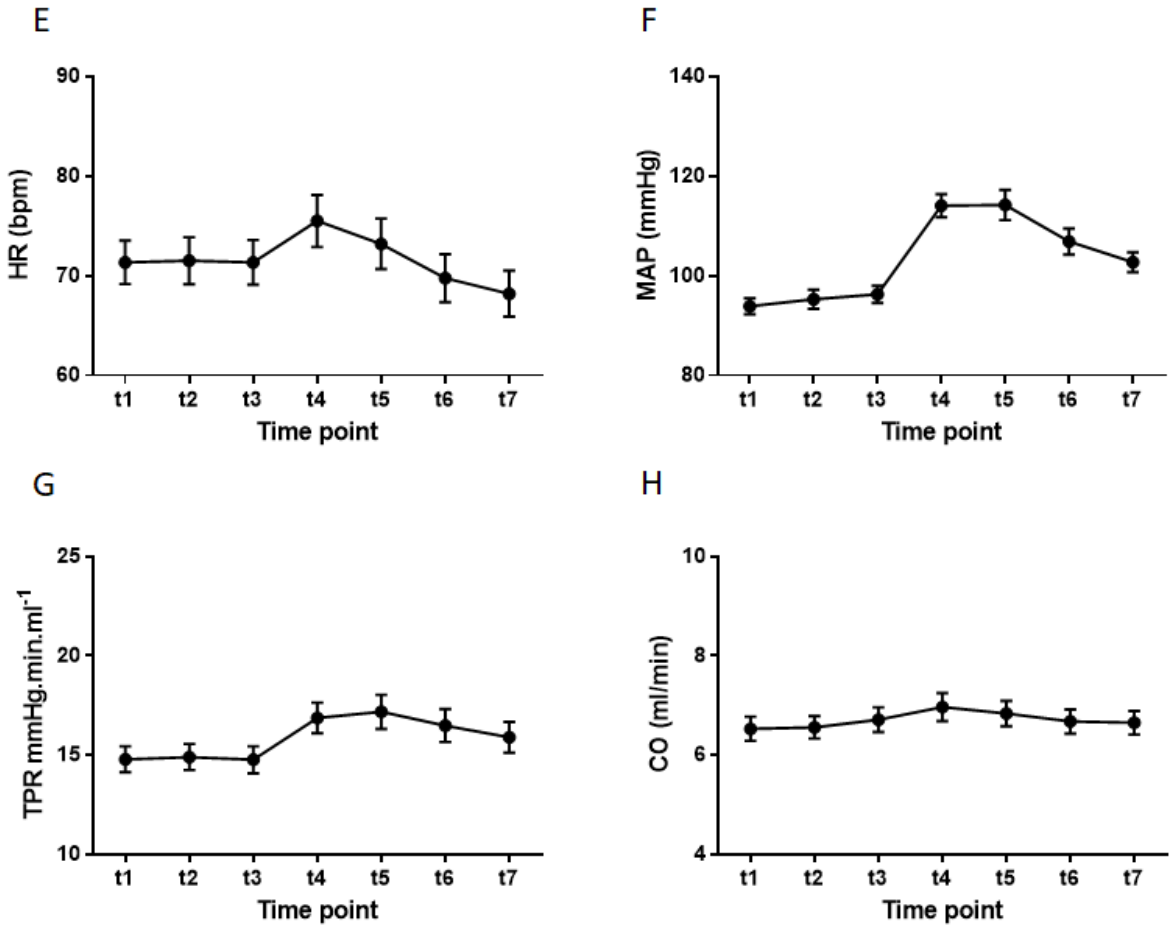
Figure 9. Normal Q-Q Plot.

Homogeneity, another assumption of the parametric analysis, was tested by the Leven's test. The mean value of SIg-A shows p= 0.89, P > 0.05, and F= 0.18 suggesting that the value is homogenous.

Appendix 2

This experiment is exploring one model of exercise, the activation of the sympathetic nervous system. Since we collected a large amount of data from the Suntech® Tango+, Nexfin®, and HIC-4000I®, we cleaned and binned the data in order to represent the 7 specific time points throughout the study. We took the saliva samples measures at 7 time-points. The time-points of all measures were averaged together at three times pre CPT, two during CPT, and two post CPT and reported as three time-points in our study. With this procedure we were able to report the underlying trends.


A
80
60
40
20
SIg-A (µg/mL)
t1
t2
t3
t4
t5
t6
t7
Time point
B
250
200
150
100
50
SαA (U/mL)
t1
t2
t3
t4
t5
t6
t7
Time point
C
160
140
120
100
SBP (mmHg)
t1
t2
t3
t4
t5
t6
t7
Time point
D
90
80
70
60
DBP (mmHg)
t1
t2
t3
t4
t5
t6
t7
Time point


E
HR (bpm)
90
80
70
60
t1 t2 t3 t4 t5 t6 t7
Time point
F
MAP (mmHg)
140
120
100
80
t1 t2 t3 t4 t5 t6 t7
Time point
G
TPR mmHg.min.ml-1
25
20
15
10
t1 t2 t3 t4 t5 t6 t7
Time point
H
CO (ml/min)
10
8
6
4
t1 t2 t3 t4 t5 t6 t7
Time point

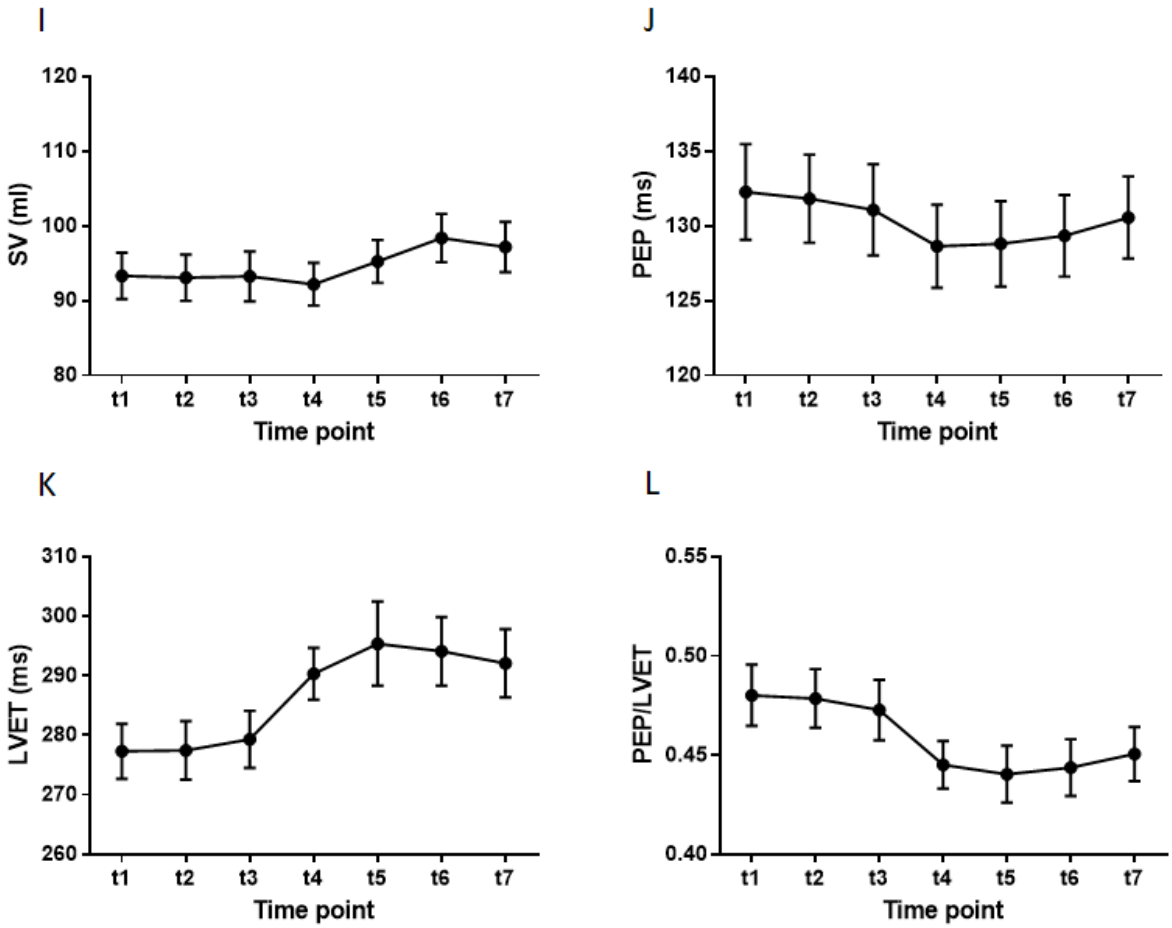


Figure 10. All measures at 7 Time-points

A) SIg-A, B) SαA, C) SBP, D) DBP, E) HR, F) MAP, G) TPR, H) CO, I) SV, J) PEP, K) LVET, and L) PEP/LVET are shown in this graph at 7 time-points. We measured t1, t2, t3 pre CPT, t4 and t5 during CPT, t6 and t7 post CPT. Bars represent SEM.

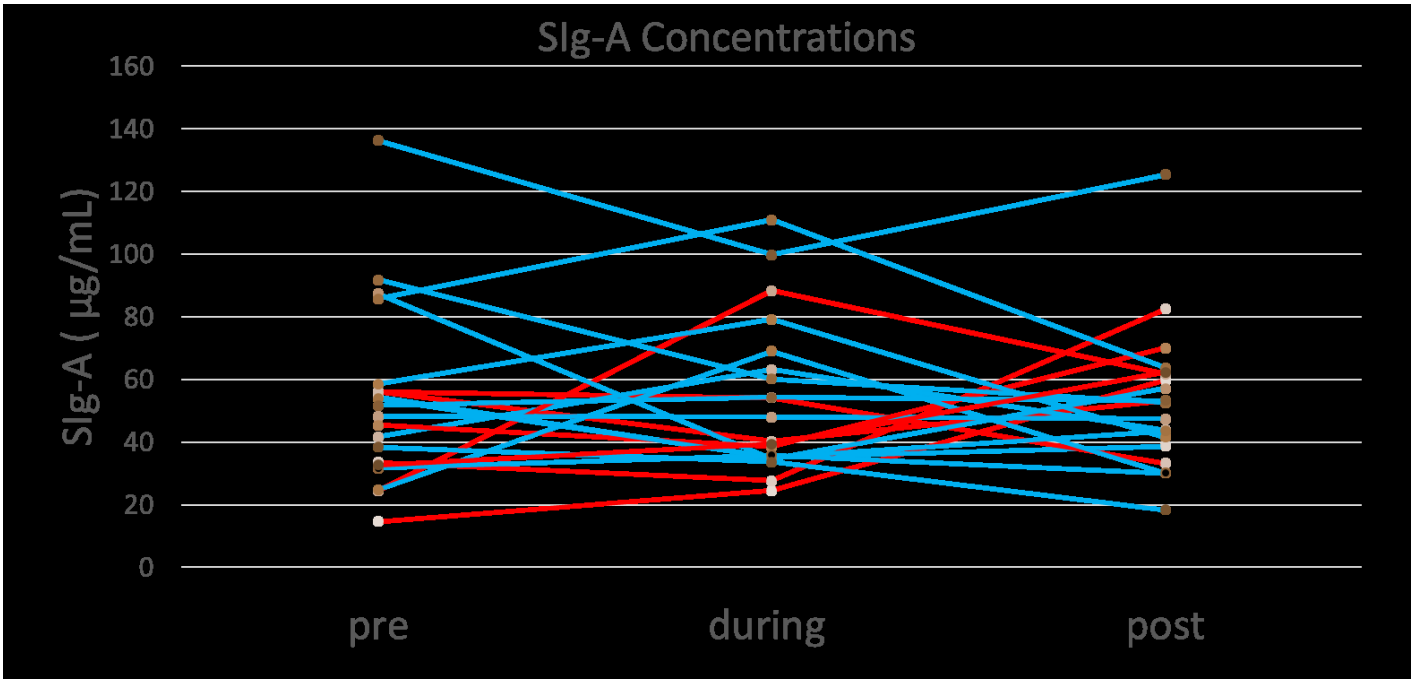


Figure 11. SIg-A Concentrations and Sex differences

The figure shows the sex difference of SIg-A concentrations at pre, during, and post concentrations. Each line represents a participant. The red lines represent females and the blue lines represent males participants.